VOLUME 1 FEBRUARY 1955 NUMBER 4

Canadian Journal of Microbiology

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

CANADIAN JOURNAL OF MICROBIOLOGY

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Canadian
Journal of Microbiology

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

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THE ANTIBODY RESPONSE IN MAN TO A QUADRIVALENT INFLUENZA VIRUS VACCINE[1]

BY BARBARA K. BUCHNER, D. B. W. REID, AND G. DEMPSTER

Abstract

The antibody response to the subcutaneous inoculation of a single 1 ml. dose of a quadrivalent formalin-killed influenza virus egg vaccine has been measured. The vaccine used contained two A prime components and an A and a B component. Satisfactory responses were obtained two weeks after inoculation to the A and B components and to one of the A prime strains (FM1). A poor antibody response was noted to the other A prime strain incorporated in the vaccine (FW50). The highest levels were obtained with the Lee strain (Type B) which also stimulated an antibody rise to a recently isolated Type B strain. Antibody levels were maintained for at least 12 weeks. Treatment of the sera with RDE was found to influence the results obtained with the FM1 strain used.

Introduction

A quadrivalent influenza virus vaccine composed of formalin-inactivated virus, harvested from the allantoic sacs of chicken embryos, was prepared by two of the writers (B.K.B. and G.D.) and was distributed throughout Canada by the Department of National Health and Welfare in the winter of 1952-53. Because of the low incidence of influenza during that period, the prophylactic effect of the vaccine could not be assessed. However, the antibody response to the vaccine was followed for a period of three months.

Four strains of virus, PR8 (A), FM1 (A prime), FW50 (A prime), and Lee (B) were included in the vaccine. The response to two of the components of the vaccine, Type A (PR8) and Type B (Lee), has been evaluated upon many previous occasions (2). Since A prime strains have been responsible for all the large epidemics in recent years, it was of particular interest to measure the effectiveness of the antibody response to the two A prime strains included in this vaccine. One of the A prime strains (FM1) has been in current use for a number of years but the other strain (FW50) has been used only recently and is closely related (3) to a strain first isolated in Canada in 1949.

In the present study the hemagglutination–inhibition (HAI) technique has been employed to measure the antibody response to the vaccine. Although

[1] *Manuscript received September 23, 1954.*
Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario.

[The December number of this Journal (Can. J. Microbiol. 1: 145-248. 1954) was issued December 10, 1954.]

Salk (7) has shown that this technique compares favorably with the virus-neutralization method, Hilleman (5) pointed out that a false estimate of the response might result with certain strains. This was due to the presence of α type non-specific inhibitor in human serum. The latter could be destroyed by preliminary treatment of the sera to be tested with the receptor-destroyer enzyme (RDE) of Burnet. A separate assessment of the antigenicity of the vaccine has been made with sera subjected to RDE treatment.

Methods

Volunteers in the field test of the vaccine were from three sources—medical students of the University of Toronto and student nurses at the Toronto Western and Women's College Hospitals. Alternate individuals were injected subcutaneously with either 1 ml. of vaccine or control fluid. Blood specimens were obtained for measurements of antibody levels from 73 of those receiving vaccine and from 78 of the control group.

The Vaccine

The influenza virus was grown in the allantoic sac of 10- to 11-day-old embryonated eggs. The four components were prepared, concentrated, and titrated separately.

Virus was concentrated in the Sharples continuous flow centrifuge. The virus concentrates were titrated according to the method recommended by Stanley (See memorandum, Federal Security Agency N.I.H., dated 1/6/49). Each component was adjusted to a strength of 500 CCA units/ml. Finally, the components were blended in the proportions: PR8 22.2%, FM1 22.2%, FW50 22.2%, and Lee 33.3%. The virus, which was suspended in phosphate buffered saline, pH 7, was inactivated by a concentration of 1 : 2,000 formalin. Merthiolate (Eli Lilly) in a final concentration of 1 : 10,000 was added as a preservative.

Control Fluid

The control fluid consisted of phosphate buffered saline, pH 7, with merthiolate at a concentration of 1 : 10,000. Formalin was added so that the percentage of free formaldehyde would be identical to that in the vaccine.

Collection and Titration of Sera

Volunteers were bled prior to the administration of vaccine or control fluid, two weeks later, and, in one group also, 12 weeks after inoculation. The sera were frozen immediately and stored at $-70°$ C. until tested for the presence of HAI antibodies. The tests were conducted on plastic plates, as described previously (3) and the value recorded was the reciprocal of the final serum dilution after the addition of all reagents. The ratio of post-inoculation to preinoculation titer was formed for each individual and the geometric mean of the ratios calculated for each group. These are reported as mean fold rises. Where the value could not be calculated because the absolute initial level was not obtained, an arbitrary value for the initial level was obtained by selecting a titer twofold lower than that tested.

RDE Treatment of Sera

A strain of *V. cholerae* designated 35A was obtained from Dr. H. Plummer, School of Hygiene, University of Toronto, and was employed for the preparation of RDE. The method of Ada and French (1) was used to prepare and concentrate the enzyme. The concentrated enzyme was diluted 1 : 40 for use and at this strength was capable, in human serum, of reducing a Francis inhibitor titer of at least 1 : 256 to less than 1 : 32. For use, the concentrated RDE was diluted in calcium acetate buffer, pH 6.0, to which 1000 units/ml. of penicillin and streptomycin had been added. A volume of 0.2 ml. of the serum was mixed with 0.6 ml. of diluted RDE and was incubated in a screw-cap vial at 37° C. overnight. For comparison a further volume of 0.2 ml. of the same serum was incubated with 0.6 ml. of acetate buffer in place of RDE and was treated with the same concentration of antibiotics. Subsequently both specimens were heated at 56-58° C. for an hour and then the HAI titer was determined. The routine method of HAI titration was modified by the use of 2% citrate saline in place of phosphate buffered saline.

Experimental

Antibody Response Two Weeks After Inoculation

Separate presentations of the results obtained with the medical students and the two groups of nurses are unnecessary since no important differences among these groups were observed. The findings presented here therefore are the combined results obtained from 73 individuals who received virus vaccine and 78 who received control fluid.

The mean HAI titers, determined initially and at two weeks, are given in Table I. In contrast to the control group, distinct elevations in titer were observed in the vaccinated group for all the strains tested. The highest mean titers recorded at two weeks were for the Lee and PR8 strains. Considerably lower levels were recorded for the A prime strains FM1 and FW50.

TABLE I

MEAN ANTIBODY TITERS

Strain	Control			Vaccinated		
	No.	Initial Titer	Titer at 2 wk.	No.	Initial Titer	Titer at 2 wk.
PR8	78	160	180	73	137	670
FM1	78	48	55	73	52	185
FW50	75	22	24	71	20	59
Lee	77	67	71	73	58	745

The mean fold rises in HAI titers during this period were calculated and are given, together with their standard errors, in Table II. In the vaccinated group, an increase of more than fourfold occurred with the Lee and PR8

strains, but weaker responses were found with the A prime strains. The mean fold rise for the FW50 strain was significantly lower than the corresponding figure for the FM1 strain. Slight changes in titer were observed in the control group, probably because of the occurrence of external stimuli in the form of subclinical infection. However, these changes were of such small magnitude that the rises recorded in the vaccinated groups may be considered as almost entirely due to the vaccine.

TABLE II

MEAN FOLD RISE AT TWO WEEKS

Strain	Control	Vaccinated
PR8	1.12 ± 0.06*	4.98 ± 0.63
FM1	1.16 ± 0.07	3.64 ± 0.45
FW50	1.10 ± 0.05	2.92 ± 0.31
Lee	1.06 ± 0.06	12.85 ± 2.07

* *Mean fold rise ± standard error.*

In Table III are shown the proportions of individuals with negative HAI titers (< 1 : 32) two weeks after inoculation. At this time a considerable proportion of individuals who had not received vaccine lacked antibody towards the FW50 and Lee strains, though only a small percentage towards the PR8 strain. The values recorded for the vaccinated group show a marked reduction for the strains FM1 and Lee. In contrast, a sizable proportion of vaccinated individuals had not developed antibody to strain FW50.

TABLE III

PERCENTAGE OF SERA WITH FINAL TITERS <1 : 32

Strain	Control	Vaccinated
PR8	2.6%	0 %
FM1	10.3%	1.4%
FW50	45.3%	18.3%
Lee	23.4%	0 %

Maintenance of HAI Titer

In those individuals who were bled 12 weeks after inoculation, the ratio of the titer at 12 weeks to the titer at two weeks was formed for each person. The mean fold rises so obtained, together with their standard errors, are shown in Table IV. They indicate little change in titer between 2 and 12 weeks. Since there was no significant change in the control group during this period, it may be assumed that the antibody response in the vaccinated group as measured at 12 weeks was virtually the same as that found two weeks after inoculation.

TABLE IV

MEAN FOLD RISE BETWEEN 2 AND 12 WEEKS

Strain	Control		Vaccinated	
	No.	Mean fold rise*	No.	Mean fold rise*
PR8	24	1.09 ± 0.07	22	1.00 ± 0.13
FM1	24	1.22 ± 0.16	22	1.17 ± 0.23
FW50	23	1.31 ± 0.15	22	1.06 ± 0.08
Lee	24	1.22 ± 0.13	22	0.97 ± 0.10

* *Geometric mean of the ratios, titer at 12 weeks/titer at 2 weeks.*

Coverage for a Recently Isolated Type B Strain (Longhway)

Further titrations to detect changes in antibody level two weeks after inoculation were made with the Longhway strain of virus and sera from 55 individuals who had received the vaccine. The sera from 56 persons who had received control fluid were tested similarly. The mean fold rise in the vaccinated group was 4.2 as compared with a value of 1.1 for the control group. Thus the vaccine stimulated a rise in titer for the Longhway strain which, although not so great as for the Lee strain, was nevertheless definite.

Assessment of the Effect of RDE

The prevaccination and two-week postvaccination sera from 27 of the 73 vaccinated persons were subjected to RDE treatment. The fold rise was determined for the RDE-treated specimen and compared with the value found with an aliquot of the same serum untreated with RDE but titrated under the same conditions. The geometric mean of the ratios so obtained was determined and is shown in Table V. The mean fold rise found after RDE treatment was significantly higher with the FM1 strain but with none other. In effect this implies that the increase in HAI titer recorded in Table II for the FM1 strain is actually greater than is shown. Thus, the real antibody response towards this strain approaches more nearly the value obtained with the Type A, PR8 strain. As the value for the FW50 strain was not altered after RDE treatment the superiority of the FM1 strain over FW50 is also greater than would appear in Table II.

TABLE V

THE EFFECT OF RDE TREATMENT UPON MEAN FOLD RISE

Strain	No.	Mean $\left(\frac{\text{Fold rise (RDE-treated)}}{\text{Fold rise (untreated)}}\right)$
PR8	27	1.20 ± 0.16
FM1	26	1.66 ± 0.11
FW50	26	1.05 ± 0.07
Lee	25	0.92 ± 0.08

Discussion

Immunity to influenza is to some extent dependent upon the blood antibody level which may be influenced readily by vaccination. In the foregoing experiments it has been possible to measure the antibody levels produced by vaccination and to show that they have been maintained for at least three months.

In attempting to assess the antibody response two independent factors which might obscure the results had to be taken into account. The first of these was the occurrence of actual infection amongst the group which might actively alter the response to a particular antigen. This possibility was adequately controlled by having within the group an equal number of persons who received only control fluid. The second factor was the difference in sensitivity of influenza strains to the presence of α type non-specific inhibitor in human serum. It was shown that this factor did affect results obtained with the A prime strain FM1.

The Type B strain (Lee) elicited a good response and as an antigen proved to have a broad enough coverage to stimulate a slightly greater than fourfold mean rise in titer to the Longhway strain. The latter strain was isolated in 1952. Thus it has been shown that the Lee component of the vaccine has produced not only a good antibody response to the standard Type B strain, but also a definite response to the recently isolated strain.

A reasonable elevation in titer (greater than fourfold) was secured for the Type A strain (PR8) and, if the effects of serum inhibitor are taken into account, the rise obtained toward the FM1 strain was also satisfactory. In comparison, the response to the FW50 strain was of a low order and examination of actual titers revealed the fact that approximately one in five individuals had an antibody level of less than 1 : 32 two weeks after vaccination.

Meiklejohn (6) has reported results with monovalent A prime FM1 vaccine and has demonstrated a certain amount of protection during an A prime epidemic. He recorded also correlation between low antibody titer as measured by HAI tests with closely related strains, and the probability of clinical infection. If we assume that a similar correlation is likely to hold for the FW50 strain, which is closely related to FM1, then the results presented in this study would suggest that the antigenic quality of the FW50 strain does not warrant its inclusion in further vaccines of this type. Francis (4) has indicated that the FM1 strain can induce cross-immunity to other A prime strains and it may be that this strain alone can provide protection against them.

Acknowledgments

The authors wish to thank Dr. C. E. van Rooyen for his advice and interest and are indebted to the members of the staffs of the School of Hygiene, The Toronto Western Hospital, The Women's College Hospital, and their students who made this work possible.

The project was supported by a grant (247) from the Defence Research Board to whom we are indebted for permission to publish.

References

1. ADA, G. L. and FRENCH, E. L. Purification of the receptor-destroying enzyme of V. cholerae. Australian J. Sci. 13 : 82. 1950.
2. BLAKE, F. G. An evaluation of vaccination against epidemic influenza in man. Bull. N. Y. Acad. Med. 24 : 308-328. 1948.
3. DEMPSTER, G. and BUCHNER, B. K. Serological pattern of influenza virus strains isolated in Canada from 1948 to 1951. Can. Med. Assoc. J. 69 : 119-124. 1953.
4. FRANCIS, T., Jr. Vaccination against influenza. Bull. World Health Organization, 8 : 725-741. 1953.
5. HILLEMAN, M. R. and WERNER, J. H. Influence of non-specific inhibitor on the diagnostic hemagglutination–inhibition test for influenza. J. Immunol. 71 : 110-117. 1953.
6. MEIKLEJOHN, G., KEMPE, C. H., THALMAN, W. G., and LENNETTE, E. H. Evaluation of monovalent influenza vaccines. II. Observations during an influenza A-prime epidemic. Am. J. Hyg. 55 : 12-21. 1952.
7. SALK, J. E., BAILEY, M. L., and LAURENT, A. M. The use of adjuvants in studies on influenza immunization. II. Increased antibody formation in human subjects inoculated with influenza virus vaccine in a water-in-oil emulsion. Am. J. Hyg. 55 : 439-456. 1952.

FACTORS INFLUENCING THE FORMALDEHYDE TREATMENT OF VIRAL ANTIGENS[1]

By John R. Polley

Abstract

An investigation has been made of the use of formaldehyde for the preparation of stable non-infective viral antigens of influenza (PR8) and mumps (Enders). The stability of the antigen to formaldehyde treatment was influenced by the pH, the suspending medium of the virus, the formaldehyde concentration, and the duration and temperature of treatment. The following procedure was found to be the most satisfactory. The supernatant liquid of allantoic fluid which had been frozen and allowed to thaw slowly was centrifuged and the virus was resuspended in isotonic phosphate buffer at pH 6.0. A hemagglutination titer of about 10,000 was attained before the process of treatment was started. This viral antigen was filtered through coarse filter paper and treated with 0.1% formaldehyde at 45° C. for 12 hr. The excess formaldehyde was neutralized by the addition of 0.25 cc. of 30% dibasic ammonium phosphate per 10 cc. of antigen. After 30 min. at room temperature, 5% (w/v) of arginine was added and the antigen was lyophilized in ampoules. These antigens have been found to be non-infective, satisfactory in the complement-fixation and hemagglutination-inhibition tests as specific diagnostic antigens, and show greater stability than liquid viral antigens.

Introduction

Formaldehyde has been used extensively for the preparation of non-infective viral and soluble antigens for the laboratory diagnosis of virus diseases. However, it has been found to render the antigens anticomplementary on storage and unsuitable for lyophilization. To overcome these difficulties, a method was developed for the preparation of stable non-infective soluble antigens of influenza and mumps (3) which is based on treating the antigens with 0.1% formaldehyde at 37° C. for two hours and neutralizing them with ammonium hydroxide prior to lyophilization. This procedure was unsatisfactory for the preparation of the corresponding viral antigens, as distinct from the soluble antigens, because it failed to destroy completely the infectivity and caused a severe loss of titer. Other reagents could be substituted for ammonium hydroxide in the preparation of soluble antigens (2) but these reagents also failed to give satisfactory results with the viral antigens. Consequently, an investigation of the use of formaldehyde in the preparation of stable non-infective viral antigens was undertaken.

Experimental Methods

The first step was an investigation of the effect of formaldehyde on the hemagglutination titer of the viral antigens to determine the extent of treatment the antigens could withstand without a serious loss of titer. The variables studied were formaldehyde concentration, suspending medium of

[1] *Manuscript received September 3, 1954.*

Contribution from the Virus Section, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada. Presented at the Fourth Annual Meeting of the Canadian Society of Microbiologists, Kingston, Ontario, June, 1954.

the virus, pH, and temperature and duration of treatment. The conditions for maximum antigen stability to formaldehyde treatment were determined. Procedures for successful lyophilization and storage of the treated antigens were also studied.

Formaldehyde Treatment

A pool of freshly harvested influenza A (PR8) virus-infected allantoic fluid was centrifuged at 19,000 *g* for one hour. The supernatant liquid was decanted and the pellets were resuspended in one third of the original volume of physiological saline, isotonic phosphate buffer at pH 7(1) or 2% albumen. Formaldehyde (10% aqueous solution) was added to samples of these viral antigens and to allantoic fluid to give concentrations of 0.5, 0.25, and 0.1% respectively. Samples of these treated antigens and untreated controls were placed in test tubes in water baths at 25°, 37°, and 45° C. Portions were removed after various times and tested for hemagglutination titer by the procedure described by Salk (4).

In Fig. 1 are shown the results obtained at 45° C. The results with 2% albumen were similar to those with allantoic fluid and the results with the saline suspension were similar to those with phosphate buffer. It can be seen that, under these experimental conditions, the hemagglutinating antigen is more stable to formaldehyde treatment when suspended in saline or phosphate buffer at pH 7 than it is in allantoic fluid or 2% albumen. The pattern of results obtained at 37° and 25° C. were similar to those obtained at 45° C. As the reaction temperature is increased, the destruction of the PR8 hemagglutinating antigen is increased and the influence of the suspending medium becomes more pronounced.

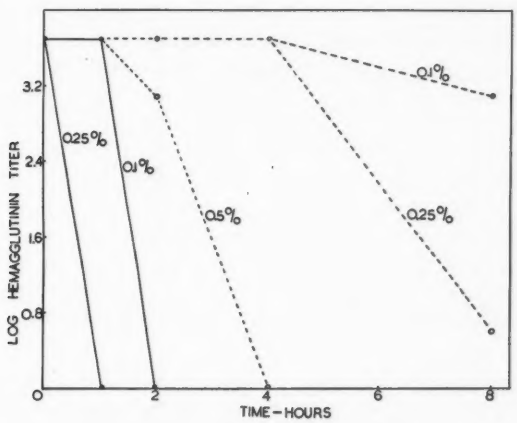

FIG. 1. Effect of suspending medium on the stability of PR8 hemagglutinin at 45° C. to various formaldehyde concentrations. ●———● Virus suspended in allantoic fluid. O - - - O Virus suspended in isotonic phosphate buffer at pH 7.

Since the stability of the hemagglutinating antigen was greater in phosphate buffer at pH 7 than in allantoic fluid, the pH of which was 8, it appeared that the pH of the suspending medium might be an important factor in determining the stability of the hemagglutinating antigen to formaldehyde treatment. To assess this possibility, PR8 viral antigens were prepared in phosphate buffers at various pH values. Formaldehyde was added to the samples to give a concentration of 0.25% in each. These samples and untreated controls were placed in tubes in a water bath at 37° C. and were tested for hemagglutination titer after various time intervals. The results (Fig. 2) indicate that, under these conditions, the stability of the PR8 hemagglutinating antigen increases as the pH is decreased and reaches its maximum at pH 6.

This experiment was repeated with samples of PR8 viral antigen suspended in allantoic fluid and in 2% albumen. Both fluids were adjusted to various pH values. Again it was found that the stability of the hemagglutinating antigen was greatest when the initial pH was 6 but the stability was less at all levels of pH than that of antigens suspended in phosphate buffer. Viral suspensions in phosphate buffer which were prepared from allantoic fluid stored at −20° C. for two years were tested in the same way and found to be as stable as those prepared from fresh allantoic fluid.

Formaldehyde Neutralization and Lyophilization

It was important to investigate methods of neutralizing the formaldehyde present after treatment because of a large loss of titer in lyophilization if free formaldehyde was present. In a previous (2) study of various reagents for the neutralization of formaldehyde in soluble antigens, it was found that dibasic ammonium phosphate, urea, sodium bisulphite, and ammonium hydroxide could be used successfully. A sample of PR8 viral antigen in

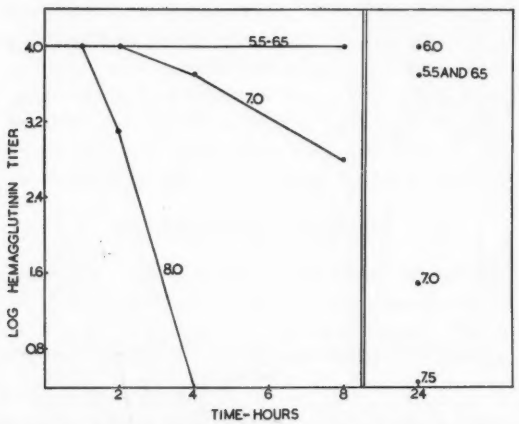

FIG. 2. Effect of pH on the stability of PR8 hemagglutinin to treatment with 0.25% formaldehyde at 37° C.

phosphate buffer at pH 6 was treated with formaldehyde. Aliquots of this treated antigen then were cooled in an ice-water bath and varying amounts of the above mentioned reagents were added. The tubes were placed at room temperature for 30 min.; the antigens were then tested for hemagglutination titer. The various antigen samples, with the exception of those treated with ammonium hydroxide, retained most of their titer after neutralization.

Samples of each of the neutralized antigens were lyophilized in an Edwards centrifugal freeze-dryer. It was found that dibasic ammonium phosphate in the form of a 30% (w/w) solution (0.25 cc. per 10 cc. of 0.1% formaldehyde-treated antigen) was the only reagent which prevented complete loss of the titer. The lyophilization was more successful when some protective material such as 0.2% albumen, 5% glucose, or 5% arginine was added. For storage of the dried antigens at room temperature, the use of 5% arginine gave the best results. It was found also that the antigen could be concentrated without causing non-specific interference in the tests by reconstituting the dried antigen to only one half of its previous volume with distilled water.

Conditions for Viral Antigen Non-infectivity

Having determined the conditions for maximal stability of PR8 hemagglutinating antigen for the three concentrations of formaldehyde used, it remained to test the final products for titer, infectivity, and specificity. The following conditions of treatment were selected for trial: (1) 0.5% formaldehyde at 45° C. for 2 hr., (2) 0.25% at 45° C. for 4 hr., (3) 0.25% at 37° C. for 8 hr., (4) 0.1% at 45° C. for 12 hr., and (5) 0.1% at 37° C. for 12 and 24 hr. Twelve pools of PR8 antigen were prepared in phosphate buffer at pH 6 and each of these pools was tested. At the end of the time of treatment, the samples were removed from the water bath and placed in an ice-water bath for five minutes. The 30% ammonium phosphate solution was added to the antigens at the rate of 0.25 cc. per 10 cc. of antigen treated with 0.1% formaldehyde (proportionately more ammonium phosphate being used for higher formaldehyde concentrations). The antigens were allowed to stand at room temperature for 30 min., after which they were lyophilized in quantities of 1 ml. per 3-ml. glass ampoule. The dried antigens were reconstituted to one-half of their previous volume with distilled water and tested for hemagglutination titer and infectivity. The infectivity tests were conducted on the reconstituted antigens by inoculating samples of them, both undiluted and after dilution 1/100, into the allantoic cavity of 10-day-old chick embryos. The eggs were incubated for a further 72 hr. Antigens that yielded fluids free of hemagglutinin after two successive allantoic passages were considered to be non-infective.

The results are summarized in Table I. All the dried antigens had titers which were satisfactory for use in the hemagglutination-inhibition test. Three of the six test groups yielded antigens which were non-infective. These antigens were tested for specificity in the hemagglutination–inhibition test. It was found that the treatment had produced no significant non-specific

reactors in some of the antigens while others showed non-specific hemagglutination to a titer of 1 : 160. The antigens were examined also in the complement-fixation test and were found to have titers of 32–64 with only negligible or no non-specific reactions.

TABLE I

TREATMENT OF PR8 VIRAL ANTIGENS WITH FORMALDEHYDE

No. of antigens tested	Treatment			Hemagglutination titer*		Infectivity
	HCHO, %	Temp., ° C.	Time, hr.	Range	Av.	
12		Controls		2560–10,240	5120	+
12	0.5	45	2	640–5120	1280	−
12	0.25	45	4	640–5120	1280	−
12	0.25	37	8	640–5120	2560	+ (1/12)
12	0.1	45	12	640–5120	2560	−
12	0.1	37	12	1280–5120	2560	+
12	0.1	37	24	640–2560	1280	+

* *Expressed as the reciprocal of the highest antigen dilution showing complete hemagglutination.*

The six procedures of formaldehyde treatment used with PR8 viral antigens were repeated with mumps (Enders) viral antigens and the results are summarized in Table II. It can be seen from this table that, with the exception

TABLE II

TREATMENT OF MUMPS VIRAL ANTIGENS WITH FORMALDEHYDE

No. of antigens tested	Treatment			Hemagglutination titer*	Complement-fixation titer**	Infectivity
	HCHO, %	Temp., ° C.	Time, (hr.)			
8		Controls		10,240	128–256	+
8	0.5	45	2	< 20	16–64	−
8	0.25	45	4	< 20	16–64	−
8	0.25	37	8	80–1280	32–64	−
8	0.1	45	12	640–2560	64–128	−
8	0.1	37	12	640–2560	32–64	+
8	0.1	37	24	320–1280	16–32	−

* *Expressed as the reciprocal of the highest antigen dilution showing complete hemagglutination.*
** *Expressed as the reciprocal of the highest antigen dilution showing 4+ fixation.*

of the 0.1% formaldehyde treatment, these methods have caused greater losses of hemagglutination titer in the mumps viral antigens than in those of influenza A. In marked contrast is the stability of the complement-fixing component of the antigen to these methods of treatment. A comparison of the hemagglutinating and complement-fixing components clearly shows the lack of relationship between them. Five of the six groups tested yielded

antigens which were non-infective. When tested for specificity in the complement-fixation test, it was shown that the treatment had produced no interfering non-specific reactors in the antigens. In the hemagglutination–inhibition test, some antigens showed no non-specific hemagglutination while others reacted non-specifically up to a titer of 1 : 160. Thus, of the methods of treatment tried, 0.1% formaldehyde at 45° C. for 12 hr. was the most satisfactory for the destruction of infectivity and the preservation, after neutralization and lyophilization, of specific antigenicity of the viral antigens of influenza A and mumps.

Non-specific Hemagglutination

It was noted in the above experiments that much of the non-specific hemagglutination of the dried antigens arose from the formaldehyde treatment itself and varied in extent with different antigens. Hence the influence of the allantoic fluid used in the preparation of the viral antigens was investigated. Viral antigens of PR8 were prepared from three different infected allantoic fluids (1) freshly harvested allantoic fluid, (2) allantoic fluid stored for three weeks at 4° C., and (3) allantoic fluid stored for various time intervals in the frozen state (the precipitate which appeared on thawing was discarded). Portions of the viral antigens were treated with formaldehyde, neutralized, 5% arginine added, and samples were lyophilized. The antigens, before and after lyophilization, and both treated and untreated, were tested for hemagglutination titer and non-specific hemagglutination. Using PR8-specific antiserum, the titers obtained with these different antigens were similar. With human serum known to give a negative reaction to PR8 virus, however, it was evident that the treated antigen prepared from freshly harvested allantoic fluid showed, after treatment, the largest amount of non-specific hemagglutination (up to a titer of 1 : 160), whereas the antigen prepared from the frozen fluid contained the least amount (up to a titer of 1 : 20).

References

1. HENDRY, E. B. The preparation of iso-osmotic phosphate buffer solutions. Edinburgh Med. J. 55 : 142-152. 1948.
2. POLLEY, J. R. Preparation of a stable noninfective soluble influenza A antigen. II. Use of various reagents for neutralization of formaldehyde-treated antigens. Can. J. Med. Sci. 30 : 194-198. 1952.
3. POLLEY, J. R., BURR, M., and GILLEN, A. Preparation of a stable non-infective soluble influenza A antigen. Proc. Soc. Exptl. Biol. Med. 76 : 330-332. 1951.
4. SALK, J. E. A simplified procedure for titrating hemagglutinating capacity of influenza virus and the corresponding antibody. J. Immunol. 49 : 87-98. 1944.

EFFECTS OF HOMOLOGOUS AND HETEROLOGOUS ANTISERA ON THE GLYCEROL DEHYDROGENASES OF HALOPHILIC AND NON-HALOPHILIC BACTERIA[1]

By R. M. Baxter and N. E. Gibbons

Abstract

Antisera against the glycerol dehydrogenases of *Escherichia coli* and *Vibrio costicolus*, prepared by injecting the enzymes into rabbits, precipitated the homologous but not the heterologous enzymes. When the enzyme from *E. coli* was completely precipitated from the supernatant by a minimal quantity of antiserum, about half the activity could be demonstrated in the precipitate. The activity of the precipitate was progressively reduced by increasing amounts of antiserum. Crude extracts of *V. costicolus* oxidized butanediol as well as glycerol. The antiserum reduced the oxidation of glycerol to a greater extent than the oxidation of butanediol indicating the presence of two enzymes in the original preparation. Antisera against the *E. coli* and *V. costicolus* enzymes were inactive against the *P. salinaria* enzyme. An attempt to prepare antibodies against the enzyme from *Pseudomonas salinaria* was not successful.

In a study of the glycerol dehydrogenases of the extreme halophile *Pseudomonas salinaria*, the moderate halophile *Vibrio costicolus*, and the non-halophile *Escherichia coli*, the only clear-cut difference between the three enzymes was the very high concentration of salts required for activity of the glycerol dehydrogenase of *P. salinaria* (2). Although the *E. coli* and *P. salinaria* preparations were specific for glycerol, crude extracts of *V. costicolus* oxidized not only glycerol but also a number of related compounds, notably D(−)-2,3-butanediol. Whether the glycerol dehydrogenase of this organism had a wider specificity than the other two or whether two or more enzymes were involved was not determined. As an extension of the comparative biochemistry of the three enzymes, some of their serological properties have been investigated.

Materials and Methods

The organisms used were as follows: *E. coli*, N.R.C. No. 482; *V. costicolus*, isolated in this laboratory from a bacon curing brine; *P. salinaria*, isolated by Lochhead (4) from salted hides. The methods for growing the organisms and preparation of cell-free extracts have been described previously (2). The *E. coli* and *P. salinaria* preparations for injection were partially purified by heat (1, 2) but this was not possible with the *V. costicolus* enzyme. Each enzyme preparation was divided into three parts; one was used for the first series of injections and the other two frozen in an alcohol – dry ice bath and stored at −20° C. for injections on subsequent weeks. The first injection was given subcutaneously, all others intravenously. Details of the procedure used in producing antisera in rabbits are outlined in Table I. Three to four weeks after the last injection the rabbits were bled, and the sera collected and stored at 4-5° C.

[1] *Manuscript received September 15, 1954.*
Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as N.R.C. No. 3443.

TABLE I

DETAILS OF INJECTION PROCEDURE

Source of extract	Treatment of extract	Protein concentration	No. of rabbits	No. of injections per rabbit	Period of injection, wk.	Total quantity injected per rabbit
E. coli	Heat and ammonium sulphate precipitation (1); made up in 0.9% NaCl	6.6 mgm./ml.	2	10	3	6.7 ml.
V. costicolus	Dialysis against 0.9% NaCl*	10.3 mgm./ml.	1	12	4**	7.4 ml.
P. salinaria	Heat and dialysis against 0.9% NaCl***	9.0 mgm./ml.	1	12	4**	8.1 ml.

* *This enzyme is less heat resistant and cannot be purified by heat.*
** *No antibody detected after three weeks.*
*** *This enzyme is inactivated by dialysis (2).*

To test antibody production, 0.1 ml. of enzyme (crude extract) was diluted when necessary and varying amounts of antiserum added to give a final volume of 2.0 ml. The *E. coli* enzyme was diluted with distilled water. Potassium chloride was added to the *V. costicolus* and *P. salinaria* preparations to give final concentrations of 0.2 and 0.8 *M* respectively. The tubes were allowed to stand at room temperature for one to two hours, then centrifuged and the supernatants decanted. Enzyme activity of suitable aliquots of the supernatants was measured in the Warburg apparatus using the ferricyanide technique (2, 6). Blanks with enzyme omitted were run through the same sequence of operations to correct for the gas production of serum alone. To determine the activity of the precipitated enzyme, the precipitate was thoroughly dispersed (*E. coli* enzyme in water; *V. costicolus* enzyme in 0.2 *M* KCl) and the activity of an aliquot of the suspension measured.

Results

Antisera of reasonable potency were prepared against the enzymes of *E. coli* and *V. costicolus*. The activity of the antiserum against the *E. coli* enzyme is shown in Fig. 1 and of the rather less potent antiserum against the *V. costicolus* enzyme in Fig. 2.

The relationship between glycerol dehydrogenase activity and antiserum concentration followed a roughly sigmoid curve for both enzymes. Small quantities of antiserum had little effect; with greater concentrations the loss of activity was approximately a linear function of the antiserum concentration until 80 to 90% had been lost. This relationship is somewhat obscured with the *E. coli* system. The sharp drop in the activity of this enzyme with low concentrations of antiserum however appears to represent a non-specific inhibition by serum, which did not occur with the *V. costicolus* enzyme. (See also Table II.)

For the butanediol dehydrogenase activity of *V. costicolus* the slope of the linear portion of the activity–antiserum concentration curve was much flatter than with the glycerol dehydrogenase of this organism.

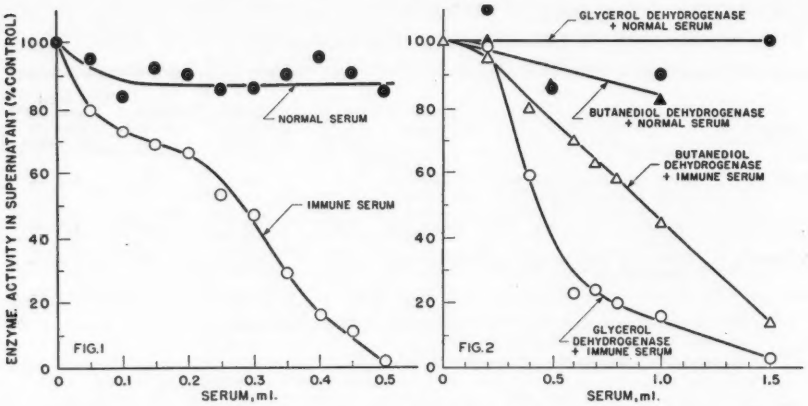

FIG. 1. Effect of specific antiserum on the glycerol dehydrogenase of *E. coli*. See text for details.

FIG. 2. Effect of specific antiserum on the glycerol and butanediol dehydrogenases of *V. costicolus*. Based on four experiments; see text for details.

When the *E. coli* enzyme was completely precipitated with approximately the minimum quantity of serum, about half of the original activity could be demonstrated in the precipitate. As the amount of serum was increased the activity of the precipitate decreased, and, with about 1.0 ml. serum to 0.1 ml. enzyme, the activity was reduced to less than one-fifth. Precipitated *V. costicolus* enzyme also showed some activity but the low potency of the serum made it impossible to demonstrate a similar inhibition with excess antiserum.

No antibody could be detected in the serum from the rabbit injected with the preparation of *P. salinaria* even after the serum had been concentrated threefold by freezing and drying. The single rabbit used may have been refractory, or the enzyme may have lost its antigenicity on inactivation by dialysis. The question was not investigated further.

TABLE II

SPECIFICITIES OF ANTISERA AGAINST GLYCEROL DEHYDROGENASES

(Figures represent activity as % of control*)

Serum \ Source of enzyme	*E. coli*	*V. costicolus*	*P. salinaria*
Normal	72	102	82
Anti *E. coli*	5	106	94
Anti *V. costicolus*	68	15	87

** Quantity of enzyme adjusted to give approximately the same activity in each. 1.5 ml. of antiserum used. Figures for normal serum average of three, others average of two experiments.*

The specificities of the antisera are shown in Table II. Under the experimental conditions employed, both the *E. coli* and the *V. costicolus* enzymes were almost completely precipitated by the homologous antiserum. However the heterologous antiserum had no more effect than normal serum and neither antiserum affected the *P. salinaria* enzyme.

Discussion

Although the *E. coli* enzyme had been partially purified, the preparations used as antigens in this investigation were undoubtedly extremely heterogeneous. It might be suggested therefore that the observed precipitation of the enzymes was the result of their adsorption on some other antigen-antibody precipitate. This criticism was applied to early studies on enzymes as antigens, but subsequent investigations make it appear invalid (cf. 5, 7). It seems safe to conclude therefore that the observed effects are the result of precipitation of the enzymes by their specific antibodies.

The failure of heterologous sera to precipitate the enzymes is in agreement with results in the literature for other enzyme systems. When cross reactions have been obtained, the use of much larger quantities of heterologous serum was required than was possible in the present study (cf. 3, 5). The results presented demonstrate that the three enzymes are different but do not permit inferences on the extent of difference. The differing effects of the antiserum against the *V. costicolus* extract on the glycerol and butanediol dehydrogenase activities of this organism indicate that two distinct enzymes are probably involved. The flatter slope of the butanediol dehydrogenase response curve probably indicates a lower titer of antibody to this enzyme.

Acknowledgment

The authors wish to thank Dr. L. Greenberg for providing rabbits and normal rabbit serum, and for helpful discussion.

References

1. ASNIS, R. E. and BRODIE, A. F. A glycerol dehydrogenase from *Escherichia coli*. J. Biol. Chem. 203 : 153-159. 1953.
2. BAXTER, R. M. and GIBBONS, N. E. The glycerol dehydrogenases of *Pseudomonas salinaria*, *Vibrio costicolus*, and *Escherichia coli* in relation to bacterial halophilism. Can. J. Biochem. Physiol. 32 : 206-217. 1954.
3. CINADER, B. Antigen–antibody interaction using enzymes as antigens. Biochem. Soc. Symposium (Cambridge, Engl.), No. 10 : 16-35. 1953.
4. LOCHHEAD, A. G. Bacteriological studies on the red discoloration in salted hides. Can. J. Research, 10 : 275-286. 1934.
5. MARRACK, J. R. Enzymes and immunology. *In* The enzymes. Vol. I. *Edited by* J. B. Sumner and K. Myrbäck. Academic Press, Inc., New York. 1950. pp. 343-389.
6. QUASTEL, J. H. and WHEATLEY, A. H. M. Anaerobic oxidations. On ferricyanide as a reagent for the manometric investigation of dehydrogenase systems. Biochem. J. (London), 32 : 936-943. 1948.
7. SEVAG, M. G. Immuno-catalysis. Charles C. Thomas, Publisher, Springfield, Ill. 1945.

A FACTOR RELEASED BY IRRADIATED CELLS THAT INDUCES RECOVERY OF VIABILITY OF ULTRAVIOLET IRRADIATED BACTERIA[1]

By Howard A. Whitehead[2]

Abstract

From an examination of the dose–survival curve of *E. coli B/r*, it was evident that the rate of survival increases with the number of cells plated as though the irradiated bacteria themselves contributed some growth restoring activity. Studies on the supernatant fluid from an irradiated suspension indicates the presence of a "restoring factor" which gives up to a fourfold increase in survival of a previously irradiated and washed suspension. The release of the "restoring factor" by irradiated cells is proportional to the rate of killing and not the degree of inactivation. The activity is apparently due to the release of some cellular constituents by living cells as a physiological response to radiation injury and not to cell disintegration products.

Introduction

In experimental studies on the biological effects of radiation, it is expected that the survival of bacteria should be greatly influenced by the treatment after irradiation. The conditions for recovery might thus yield some information as to the nature of the irradiation process. Some recovery processes which have been demonstrated in ultraviolet irradiated bacteria are photoreactivation of the lethal and mutagenic effects in *Escherichia coli B/r* (6, 7, 15, 16), heat reactivation of *E. coli* "*B*" (2), catalase restoration in *E. coli* K-12 (9, 13, 14), and peroxidase restoration in *E. coli* K-12S (8). The present report deals with a type of restoration process in *E. coli B/r* in the form of a "restorative factor" released by irradiated cells as a physiological response to injury.

Materials and Methods

Escherichia coli strain *B/r*, a radiation resistant strain derived from the sensitive parent "B" (18) was employed as both the organism injured and the test organism.

Cultures were grown in Difco nutrient broth at 37° C. with aeration, and the cells harvested after 12 hr. growth. At this time the bacteria were still highly active metabolically, having just completed the logarithmic phase of growth, and showed a maximum cell count. The cells were washed three times in 0.85% saline by centrifugation and between washings were vigorously shaken on an electric shaking machine ($\frac{1}{4}$ in. elliptical stroke at 1300 cycles per minute) to help remove any material which might have adhered to the cells and to break up any cell aggregates which might form. The thrice washed cells were then suspended in M-9-1 buffer solution (1) at pH 7.0 and

[1] *Manuscript received September 29, 1954.*
Contribution from the Department of Bacteriology and Immunology, McGill University, Montreal, Quebec.
[2] *Present address: Research and Development Center, Kimberly-Clark Corp., Neenah, Wisconsin, U.S.A.*

standardized on the Evelyn photoelectric colorimeter to give a suspension containing 3.0×10^9 cells per ml. All viable count estimations were determined on beef heart infusion agar by the 'drop plate' method using calibrated pipettes (17).

Irradiation

The ultraviolet source was a 15 watt General Electric Germicidal Lamp, a low pressure discharge lamp estimated to emit 95% of its radiant energy in the 2537 Å line. Dosage has been indicated by the time of exposure in seconds with the lamp source 38.5 cm. above the surface of the suspension. Ten-milliliter samples were irradiated in special plane bottomed assay dishes (Corning Glass Works, Corning, N.Y.) of 100 mm. diameter and 20 mm. depth. Samples of this volume in the dishes used gave a depth of 1.75 mm. for irradiation. During the irradiation process the samples were shaken mechanically to ensure uniform exposure of all cells. The irradiation procedure was carried out in a darkened room to avoid the possibility of photoreactivation.

Experimental

When a homogenous suspension of *E. coli B/r* is subjected to increasing dosage of ultraviolet light, the dose–survival curve obtained is sigmoid in shape, and the rate of survival increases with dose. Since with decrease in survival there is an increased number of cells plated, this might be due to *(a)* some factor(s) released by the dead or injured cells into the suspending fluid as a physiological response to injury, or *(b)* to the presence of the dead cells themselves which might adsorb toxic substances or slowly release some active factor. Delaporte (3, 4) has demonstrated that cells of *E. coli B* plated on nutrient agar, ultraviolet irradiated, and incubated show different growth responses depending on the density of the plated aliquot. Survival is greatest among cells plated in groups or masses than with single cells and the greater proximity of one cell to another also enhances recovery. That there is some restoring factor released by cells upon irradiation is demonstrated in Fig. 1 where curve A represents a standard cell suspension which has been irradiated and plated in the normal manner for viable cell counts. Curve B represents a suspension which has been irradiated and the cells washed and resuspended in fresh buffer for plating. Washing and resuspending was carried out in a darkened cold room. It would appear that in curve B the active restoring factor released by the cells upon irradiation has been removed by washing and resuspension.

It can be shown that the "restorative factor" contained in an irradiated suspension is present only in the supernatant fluid from the suspension and is not due to the presence of dead cells. The cell-free supernatant fluid, which consists of the suspending medium and the "restorative factor" will be referred to as the "active supernate". Table I demonstrates the increase factor due to an active supernate of a second irradiated and washed suspension of *E. coli B/r* on a standard irradiated test sample compared with the

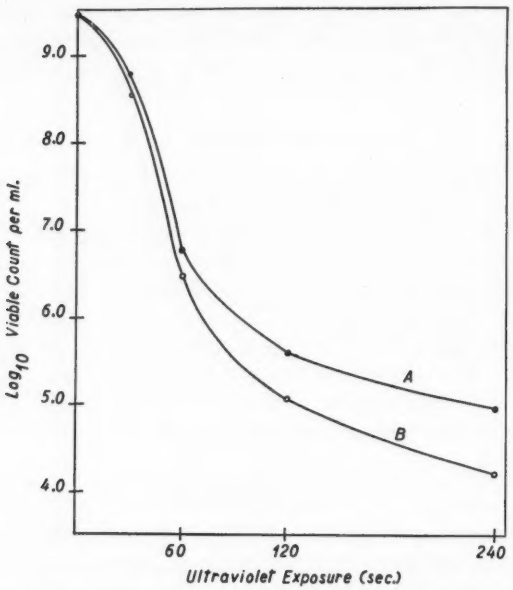

FIG. 1. Dose survival curves for *Escherichia coli B/r*. A. Cells irradiated and plated in an "active supernate". B. Cells irradiated and washed; plated in buffer.

TABLE I

PARTIAL RESTORATION OF ULTRAVIOLET IRRADIATED *E. coli B/r* BY AN ACTIVE SUPERNATE, DEAD CELLS, AND PHOTOREACTIVATION

Ultraviolet exposure (sec.)	Increase factor due to		
	Active supernate	Killed cells	Photo-reactivation
0	0	0	0
120	2.1	0	5.9
240	4.7	1.0	12.3

increase factor obtained with a similar suspension treated after irradiation with irradiated killed cells only; both are compared with the increase obtained by photoreactivation.

The increase factor obtained with the active supernate is only one-third that obtained with photoreactivation, but like the latter phenomenon the effect increases with increasing inactivation dose. Failure to obtain as high a degree of restoration may be due to the production of some toxic products in the supernatant fluid as well as growth stimulating products (12).

It would appear that the "restorative factor" is released by cells as a physiological response to injury. In this respect it would be like the "proliferation-promoting hormones" released by injured animal tissues described by

TABLE II

IRRADIATED AND UNIRRADIATED CELL-FREE FILTRATE RESTORATION OF ULTRAVIOLET INACTIVATED *E. coli B/r*

Ultraviolet exposure (sec.)	Survival, %			
	Irradiated control	Cells plated in diluent of		
		Active supernate	Supernatant from an unirrad. susp.	Irrad. super. from unirrad. suspension
0	100	100	100	100
120	5.5×10^{-2}	1.1×10^{-1}	5.6×10^{-2}	5.5×10^{-2}
240	3.2×10^{-3}	9.7×10^{-3}	3.1×10^{-3}	3.2×10^{-3}

Loofbourow *et al.* (11, 10). No increased cell count is obtained with an irradiated washed suspension when the cells are placed in cell-free filtrates from a stored unirradiated suspension nor from such filtrates that have been irradiated.

The release of the "restorative factor" by irradiated cells is greatly influenced by the rate and not the degree of killing. A comparison was made of the effect of rapid and slow killing on the release of "restorative factor". It was found that the release of the factor increased with the time required to kill the cells but was independent of the number of cells killed. This would indicate that the "restorative factor" is not due to cell disintegration products but is a physiological response to injury, probably released through an increased membrane permeability. Microscopic examination of irradiated cells shows no evidence of cytolysis. Table III indicates the increase factor by active supernates released by 30, 60, and 120 min. of irradiation to obtain both 50% and 1×10^{-1}% viability.

The release of the "restorative factor" is not limited to the homologous species but can be obtained from other injured cells only to a lesser degree of activity. The increase in survival of irradiated *E. coli B/r* suspensions by

TABLE III

DEGREE AND RATE OF KILLING ON THE RELEASE OF THE RESTORATIVE FACTOR BY IRRADIATED *E. coli B/r*

Ultraviolet exposure (sec.)	Increase due to 'restorative factor' released by irradiation for		
	30 min.	60 min.	120 min.
To obtain 50% viability			
0	0	0	0
120	1.4	1.6	2.2
240	3.2	4.5	5.1
To obtain 1×10^{-1}% viability			
0	0	0	0
120	1.7	1.8	2.6
240	3.9	4.5	5.9

substances released from irradiated *Saccharomyces cerevisiae* suspensions (10 gm. per 100 ml.) was only one-half of that obtained by the active supernate from irradiated *E. coli B/r*, and that from minced liver (30 gm. per 100 ml.) exhibited less than one-third activity.

The active supernate containing the "restorative factor" is non-protein in nature, giving a negative Biuret test, and does not contain nucleic acid, being insensitive to Bial's reagent. The pH of the fluid is neutral. Attempts have been made to simulate the action of the active factor with known growth factors, amino acids, nucleic acids and their hydrolytic products, catalase, ATP, DPN but no positive results have been obtained.

The "restorative factor" obtained might be similar to the yeast and meat extracts recently described by Hollaender *et al.* (5) which reduces the radiation damage in X-irradiated *E. coli*, although a simultaneous comparison of the two effects would have to be carried out before a statement could be made.

References

1. ANDERSON, E. H. Growth requirements of virus-resistant mutants of *Escherichia coli* strain "B". Proc. Natl. Acad. Sci. U.S. 32 : 120-128. 1946.
2. ANDERSON, E. H. Heat reactivation of ultraviolet-inactivated bacteria. J. Bacteriol. 61 : 389-394. 1951.
3. DELAPORTE, B. Action réciproque excitatrice de croissance de cellules bactériennes contiguës préalablement exposées aux rayons ultraviolets. Compt. rend. 232 : 1378. 1951.
4. DELAPORTE, B. and DELAPORTE, P. J. Etude statistique des facteurs de croissance lies a la proximite des bactéries entre elles. Riassunti delle Communicazions. VI Congr. Intern. Microbiol., Roma, I : 49. 1953.
5. HOLLAENDER, A., STAPLETON, G. E., and BILLEN, D. The reduction of radiation damage to living cells by treatment after exposure. Science, 117 : 468. 1953.
6. KELNER, A. Photoreactivation of ultraviolet-irradiated *Escherichia coli* with special reference to the dose reduction principle and to ultraviolet induced mutation. J. Bacteriol. 58 : 511-522. 1949.
7. KELNER, A. Light-induced recovery of microorganisms from ultraviolet radiation injury with special reference to *Escherichia coli*. Bull. N.Y. Acad. Med. 26 : 189-199. 1950.
8. LATARJET, R. Action restauratrice de la peroxydase sur des bactéries irradiées. Compt. rend. 235 : 1701-1702. 1952.
9. LATARJET, R. and CALDAS, L. R. Restoration induced by catalase in irradiated microorganisms. J. Gen. Physiol. 35 : 455-470. 1952.
10. LOOFBOUROW, J. R., DWYER, C. M., and CRONIN, A. G. Proliferation-promoting intercellular hormones. 2. Evidence for their production by living cells as a response to injury. Biochem. J. (London), 35 : 603-609. 1941.
11. LOOFBOUROW, J. R., DWYER, C. M., and LANE, M. M. Proliferation-promoting intercellular hormones. 1. Quantitative studies of factors produced by injured animal tissue cells. Biochem. J. (London), 34 : 432-441. 1940.
12. LOOFBOUROW, J. R. and MORGAN, M. N. Investigation of the production of growth-promoting and growth-inhibiting factors by ultraviolet irradiated microorganisms. J. Bacteriol. 39 : 437-453. 1940.
13. MILETIC, B. and MORENNE, P. Nouvelles recherches sur la restauration induite par la catalase chez des bactéries irradiées. Ann. inst. Pasteur, 83 : 515-526. 1952.
14. MONOD, J., TORRIANI, A. M., and JOLIT, M. Sur la reactivation de bactéries sterilisées par la rayonnement ultraviolet. Compt. rend. 229 : 557. 1949.
15. NEWCOMBE, H. B. and WHITEHEAD, H. A. Photoreversal of ultraviolet-induced mutagenic and lethal effects in *Escherichia coli*. J. Bacteriol. 61 : 243-251. 1951.
16. NOVICK, A. and SZILARD, L. Experiments on light-reactivation of ultraviolet inactivated bacteria. Proc. Natl. Acad. Sci. U.S. 35 : 591-600. 1949.
17. REED, R. W. and REED, G. B. "Drop plate" method of counting viable bacteria. Can. J. Research, E, 26 : 317-326. 1948.
18. WITKIN, E. Inherited differences in sensitivity to radiation in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S. 32 : 59-68. 1946.

YELLOW CHROMOGENIC BACTERIA ON WHEAT

I. QUANTITATIVE STUDIES[1]

By Norman James

Abstract

Yellow chromogenic bacteria, of the type previously reported on commercial samples, were found on all samples representing five varieties of wheat grown at 21 stations in 1952, and six varieties grown at 19 stations in 1953. Numbers varied widely, significantly from station to station and from year to year, but not significantly from variety to variety. A correlation between numbers of bacteria and yield of wheat was apparent from the data, but the coefficient was not significant at $P = 0.05$.

Introduction

According to a report from this laboratory (2) wheat passing through the Winnipeg market in 1943 and in 1945 harbored large numbers of bacteria. The number was smallest on No. 1 Manitoba Northern red spring wheat and progressively larger on each successive lower grade. Even after the grain had been washed vigorously 12 times in sterile water, four crushed samples of No. 1 Manitoba Northern gave counts ranging from 49,000 to 630,000 per gm. Vitreous kernels selected from a sample of No. 4 Manitoba Northern harbored the smallest number of bacteria, with weathered, immature, and frosted kernels sustaining progressively larger numbers. About one third of the surface colonies developing on nutrient agar plates were yellow; and on the basis of colony characteristics and size, shape, motility, and Gram's reaction of cells appeared to represent one species. In a later study Stark (3) reported that bacteria on growing wheat plants at the pre-heading stage were relatively few in numbers and represented several types, whereas on ripe and overripe plants numbers were large and the predominant type was the yellow chromogen referred to in the earlier report. This investigator reported also on the flora of commercial flax, barley, and oats. These grains likewise harbored large numbers of bacteria; and the yellow chromogen was in much larger numbers than any other type.

The present report deals with the incidence of these yellow chromogens on different varieties of wheat produced in different areas of the three prairie provinces of Canada under different soil and climatic conditions.

A later paper in this series will deal with the problem of classification. In the original report from this laboratory (2) the opinion was expressed that this chromogen represented an epiphyte of a wide variety of plants to which Düggeli (1) early gave the name *Bacterium herbicola aureum.*

Materials and Methods

Samples for this study and data on yields of wheat were provided through the courtesy of the staff of the Laboratory of Cereal Breeding, Federal Depart-

[1] *Manuscript received October 4, 1954.*
Contribution from the Department of Microbiology, The University of Manitoba, Winnipeg, Man., with financial assistance from the National Research Council of Canada.

ment of Agriculture, located at the university. They represented a comprehensive major undertaking by 23 co-operating agencies. Tests on yields, specific diseases, and certain agronomic factors were conducted on wheat varieties at stations grouped on a regional soil-climatic basis, as follows: Manitoba and black soil zones of Saskatchewan, seven stations; brown soil zones of Saskatchewan and Alberta, twelve stations; and black and gray soil zones of Alberta, four stations. Each test consisted of a rectangular lattice experiment with five plots per block and six replications of 30 varieties. Only five of these varieties were used in this study in 1952, and the same five and one other variety in 1953. Further, only 21 stations could be considered in 1952, and 19 stations in 1953. Eighteen of these were the same for the two years. Samples from the other stations were not available because of crop failure at these points due to hail, drought, or other crop hazard. Our laboratory received the samples within a few days of threshing and counts were made within a month, the samples investigated at one time being selected at random. The laboratory procedure has been outlined (2). The counts recorded in this study represent typical yellow surface colonies, and not the total population developing on nutrient agar.

Results

Numbers of yellow chromogenic bacteria in washings from the 105 samples harvested in 1952 ranged from 0 to 380,000 per gm. On the few samples where the numbers were low the total bacterial population was low also. On samples with medium numbers the yellow chromogen accounted for about one half of the population; and on the few samples where numbers were high the dilutions selected for counting contained the chromogen only. The differences between stations were readily recognizable; whereas the differences between varieties from any station were small. The data on the 114 samples harvested in 1953 were quite like those on the 1952 samples. However, it was apparent that the relation between numbers and stations was not the same for the two years. The relation between bacteria and stations, varieties, and years is shown in Table I. For this study data from 18 stations and five varieties were available for the two years.

TABLE I

ANALYSIS OF VARIANCE OF THE EFFECT OF STATION, VARIETY, AND YEAR ON THE NUMBER OF YELLOW CHROMOGENIC BACTERIA ON WHEAT

Source of variance	Degrees of freedom	Mean square	*F*
Stations	17	9.8818	21.4915**
Varieties	4	0.9683	2.1059
Years	1	20.3683	44.2981**
Stations × varieties	68	0.4541	0.9876
Stations × years	17	2.5775	5.6056**
Varieties × years	4	0.2626	0.5711
Stations × varieties × years = (error)	68	0.4598	—

** *Significant at P = 0.05.*

Since the number of stations in the three regional and soil zones differed widely, a statistical study of numbers of bacteria in relation to soil zone was not made. This relationship is shown in Table II. A large proportion of the samples grown in the brown soil zones of Saskatchewan and Alberta harbored only relatively small numbers of these bacteria, whereas most samples grown in the other two regional soil zones harbored much larger numbers.

TABLE II

EFFECT OF REGION AND SOIL ZONE ON NUMBERS OF YELLOW CHROMOGENIC BACTERIA ON WHEAT

Region and soil zone	Samples tested	% under 10,000	% over 100,000
Manitoba and black soil zones of Saskatchewan	65	15	34
Black and gray soil zones of Alberta	30	16	63
Brown soil zones of Saskatchewan and Alberta	110	63	13

Forty pairs of data on average numbers of bacteria for the five varieties (six in 1953) and average yield per acre for the same varieties in the two-year period were used in correlation studies. These are shown in Fig. 1. The correlation coefficient for these data is 0.262 which is not significant at $P = 0.05$.

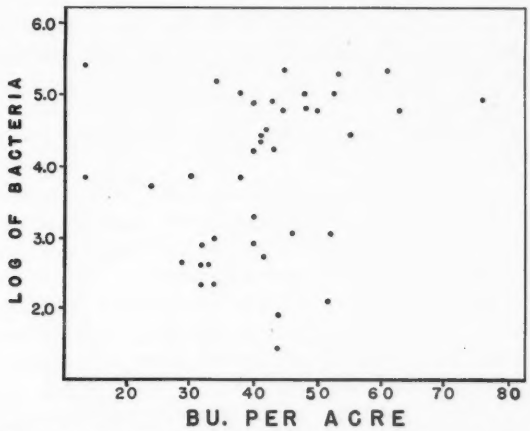

FIG. 1. Relationship between yields and numbers of yellow chromogenic bacteria on wheat.

Discussion

The data presented provide additional evidence that wheat is the habitat of this group of yellow chromogenic bacteria. These bacteria were present on all varieties and at all stations each year. Their numbers varied widely. However, the data are not adequate to show what factor, or group of factors,

was responsible for this variation. It is apparent from Table II that numbers are different in different regional soil zones; but the relationship is not consistent. A considerable proportion of the samples from each soil zone harbored numbers of bacteria differing widely from numbers on the majority of samples. In the data presented in Table I the soil zone factor was constant, since the tests were conducted at the same 18 stations in the two years. The large mean square for years indicates that factors other than soil type and variety are responsible for the differences in numbers of bacteria in the two years.

Since the procedure followed for obtaining an estimate by the plate method was an adaptation wherein only typical colonies on the surface of the agar medium were counted, it might be considered that the failure to obtain better evidence of correlation of numbers with the factors studied could be attributed to inadequacy of the method. However, the relatively small difference in numbers between varieties at most stations, as shown by the small mean square for varieties in Table I, justifies acceptance of the method for the purpose used. This regular close agreement among estimates on different varieties scarcely could be considered a chance finding.

Acknowledgment

The technical assistance of Eugene Roslycky and Ivan Kochan is gratefully acknowledged.

References

1. DÜGGELI, M. Die Bakterienflora gesunder Samen und daraus gezogener Keimplanzchen. Centr. Bakteriol., Abt. II, 13 : 56-63 and 198-207. 1904.
2. JAMES, N., WILSON, J., and STARK, E. The microflora of stored wheat. Can. J. Research, C, 24 : 224-233. 1946.
3. STARK, E. The microflora of cereals. M.Sc. Thesis, University of Manitoba, Winnipeg, Man. 1948.

CANDIDA MALICOLA, NOV. SP., ISOLATED FROM APPLES[1]

By D. S. Clark and R. H. Wallace

Abstract

A new non-fermenting species of *Candida* has been isolated from apples. It has apiculate lemon-shaped cells which have the ability to assimilate many of the common sugars, except lactose. It is similar physiologically to *C. scottii* but it differs morphologically so far as to justify a new specific name, *Candida malicola*.

During a recent study of the microflora of apples (1) it was noted that about 75% of the yeasts appearing on the agar medium used for plating seemed to be of one kind. Cells from 25 colonies were isolated and the resulting cultures were tested for purity. Studies of the morphological and physiological characteristics of these cultures showed that they appeared to be a species of *Candida* that hitherto has been undescribed. The name *Candida malicola* is proposed, from the Latin '*malum*' meaning 'apple', and '*colo*' meaning 'inhabit'.

Candida malicola, nov. sp.

As shown in Fig. 1 the cells are lemon-shaped and apiculate, varying from 3.1 to 10.5 μ in length and from 1.8 to 4.6 μ in width; they have polar and bipolar budding in which the buds are either single or paired. During its growth on agar the yeast forms an abundance of septate mycelia (Fig. 2) with oval and apiculate blastospores (Fig. 3). The colonies on Yeast Morphology Agar (Bacto) have smooth, dull, cream-colored surfaces; they have a butyrous consistency and are surrounded by a growth of mycelium under the surface of the agar. In broth media the cells form a thin pellicle that breaks very easily.

The yeast does not ferment any of the common sugars, but assimilates galactose, glucose, maltose, mannose, raffinose, sucrose, and trehalose; it also assimilates ethyl alcohol, mannitol, and sorbitol. It does not assimilate lactose. As a source of nitrogen it uses any of the following compounds: ammonium chloride, ammonium nitrate, ammonium phosphate, ammonium sulphate, peptone, urea, amino acids, and yeast extract. It liquefies gelatin slowly, and causes a peptonization of litmus milk when incubated for seven days at room temperature. It does not split arbutin nor form starchlike compounds or carotenoid pigments.

The yeast grows best at 22–25° C. with the pH of the medium between 4.9 and 6.8. The thermal death time is 16 min. at 40° C., eight minutes at 42° C., and two minutes at 44° C. The yeast does not seem to be affected adversely by the usual preservatives since it grows well in nutritional media containing 1% sodium benzoate, or lactic acid, or boric acid.

[1] *Manuscript received October 25, 1954.*
Contribution from the Department of Agricultural Bacteriology, Macdonald College, McGill University, Montreal, Quebec. Macdonald College Journal Series No. 361.

TABLE I

CHARACTERISTICS OF *C. malicola* COMPARED WITH OTHER NON-FERMENTING *Candida* SPECIES

Species	Morphology	Assimilation: Glucose	Maltose	Galactose	Lactose	Saccharose	KNO_3	Litmus milk	Arbutin split
C. mesenterica	Oval–elongate	+	+	−	−	+	−	No change	+
C. humicola	Elongate	+	+	+	+	+	−	Coagulation and peptonization	+
C. rugosa	Oval–cylindrical	+	−	+	−	−	−	Blue	−
C. zeylanoides	Oval–long	+	−	−	−	−	−	Blue	−
C. lipolytica	Long-oval–elongate	+	−	−	−	−	−	Peptonization	−
C. japonica	Round–oval	+	+	−	−	+	−	Coagulation	+
C. scottii	Long-oval–elongate	+	+	+	−	+	+	Peptonization	+ −
C. curvata	Oval–curved	+	+	+	+	+	−	Coagulation	−
C. malicola	Lemon-shaped and apiculate	+	+	+	−	+	+	Peptonization	−

Table I shows the characteristics of the *Candida* species described by Lodder and Kreger-van Rij (2) that do not ferment sugars; some of the characteristics of *C. malicola* also are shown for comparison. The carbon and nitrogen requirements of *C. malicola* are similar to those of *C. scottii*; the effects of the two species on litmus milk and arbutin are also similar. The basic differences between the two species are, therefore, in their morphological characteristics. *C. scottii* is described (2) as having long-oval to elongate cells, whereas *C. malicola* has mostly apiculate lemon-shaped cells that do not develop into elongate forms; *C. scottii* forms pseudomycelia, whereas *C. malicola* has septate mycelia. Furthermore, *C. scottii* forms a ring and occasionally some islets on liquid media, but *C. malicola* forms a thin pellicle.

A culture of *C. malicola* has been sent to the American Type Culture Collection, Washington, D.C., U.S.A.; others are being stored in the Macdonald College Culture Collection.

Acknowledgment

The authors are indebted to the Scientific Research Bureau, Quebec Department of Trade and Commerce, for assisting this work financially; and to Miss J. Audrey Williams, B.Sc. (McGill), for confirming many of the tests.

References

1. CLARK, D. S. Taxonomy of yeasts isolated from apples. M. Sc. Thesis, McGill University, Montreal, Que. 1953.
2. LODDER, J. and KREGER-VAN RIJ, N. J. W. The yeasts. A taxonomic study. North-Holland Publishing Co., Amsterdam. 1952.

[Plate I faces this page.]

PLATE I

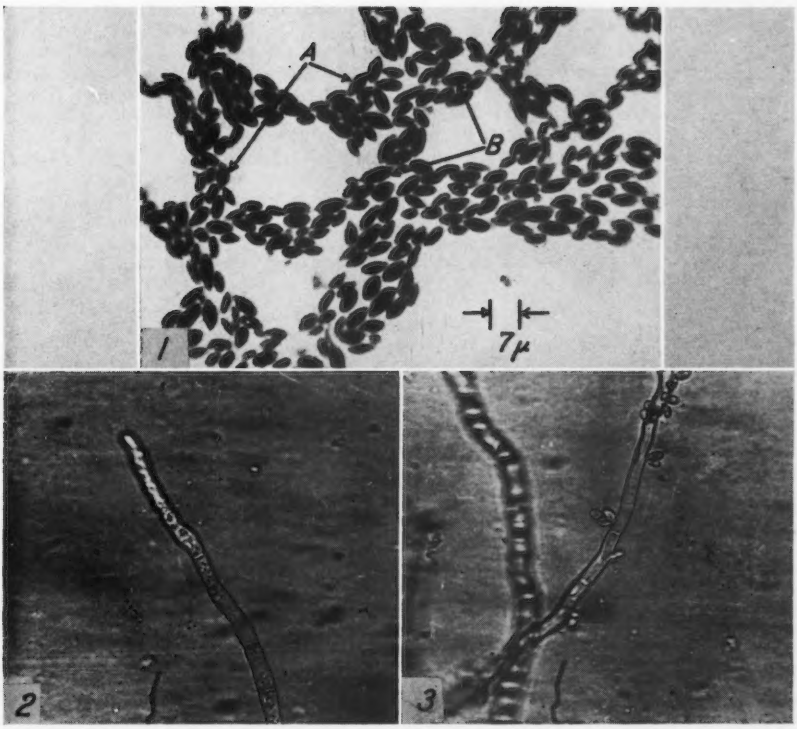

FIG. 1. Typical cells of *C. malicola*, with lemon-shaped forms (*A*) and bipolar budding (*B*).
FIG. 2. A growing tip and septa of the mycelium of *C. malicola*. Magnified 2730 ×.
FIG. 3. Blastospores growing on the mycelium of *C. malicola*. Magnified 2730 ×.

ESCHERICHIA COLI STRAINS ASSOCIATED WITH GASTRO-ENTERITIS IN INFANTS WITH SPECIAL REFERENCE TO A HOSPITAL OUTBREAK DUE TO *E. COLI* TYPE 111;B4[1]

By V. Crossley, M. Simpson, and M. Finlayson

Abstract

Since 1952 specimens of stool from infants have been examined for *Escherichia coli* types 111;B4 and 55;B5. In 1953 the examination of these specimens was extended to include *E. coli* types 26;B6, 86;B7, 125, and 126. *E. coli* type 111;B4 was identified 156 times from 138 infants varying in age from newborn to one year. These strains could be separated into the three serological groups described by Kauffmann. There were 33 of subtype 111;B4;2, six of 111;B4;12, and 103 non-motile strains without H antigen. The 111;B4;2 cultures were salicin negative and resistant to streptomycin, whereas 111;B4;12 cultures were salicin positive and sensitive to streptomycin. Subtype 111;B4;2 was responsible for a hospital outbreak of gastro-enteritis in which the organism was isolated from the feces of 25 infants. Isolations of the five other types totalled 18 from 15 patients. Ninety-five per cent of the patients from whom the six *E. coli* types were isolated had a history of diarrhea of more or less severity.

Introduction

In 1945 Bray (1, 2) described a strain of *Escherichia coli* associated with summer diarrhea in infants under the name *Bacterium coli neapolitanum.* Numerous reports of *E. coli* strains associated with hospital outbreaks of gastro-enteritis in infants have appeared in the literature both in Europe and on this continent (3, 4, 5, 6, 7, 8, 9, 10).

In 1951 and 1952 antisera were prepared in this laboratory for *E. coli* types 111 ; B4 and 55 ; B5. From the time the sera were completed, all specimens of stool received from infants of one year or younger were examined for these types of *E. coli.* In 1953 antisera were prepared for types 26 ; B6, 86 ; B7, 125, and 126 as well as a pooled OB serum of the six types, and the routine examination of infant fecal specimens was extended to include these new types.

In the two year period during which stool specimens from infants have been examined for *E. coli* types 111 ; B4 and 55 ; B5 and in the nine month period since types 26 ; B6, 86 ; B7, 125, and 126 were included in the examination, one or more of each of these types and one hospital outbreak have been encountered.

Sources of Strains

Table I shows the number and distribution of the types isolated. Diarrhea of more or less severity was reported on 95% of the patients from whom these *E. coli* types were isolated. Clinical information was not available on the remaining 5%.

[1] *Manuscript received October 8, 1954.*

Contribution from the Central Laboratory, Ontario Department of Health, Toronto, Ontario.

TABLE I

NUMBER AND DISTRIBUTION OF TYPES ISOLATED

Type	Number of cultures	Number of patients	Number of locations
111;B4	156	138	8
55;B5	4	4	2
26;B6	8	5	3
86;B7	1	1	1
125	1	1	1
126	4	4	1
	174	153	16

During 1952 and the early part of 1953, 111;B4 had been identified 24 times from 21 patients in seven widely separated districts, but on Nov. 16, 1953, *E. coli* type 111;B4 was isolated from four infants in one hospital, which we will refer to as hospital *A*. A second hospital, hospital *B*, was later involved in the outbreak. Between Nov. 16 and Dec. 7, 39 stool specimens were received from both the nursery and pediatric wards of Hospital *A*. From 12 of these specimens, *E. coli* 111;B4 was isolated, making a total of 16 cases up to that time.

As a result of these findings, a doctor and a nurse from the Division of Maternal and Child Hygiene and a medical bacteriologist from the Laboratory Division visited hospital *A* on Dec. 10, 1953. Fecal specimens, anal swabs, and nasal swabs were received from all infants that were in the hospital at that time. The nasal swabs were uniformly negative for *E. coli* 111;B4, as were those also from the nurses and ward aids. A few stool specimens that were received from the nurses and mothers were also negative. No *E. coli* 111;B4 was found on swabs of various locations in the nursery, pediatric ward, or formula room. Table II shows the number of patients and the age of the infants from whom the organism was isolated. It will be noted that one of these infants was one year of age.

TABLE II

AGE GROUPS OF PATIENTS IN HOSPITALS *A* AND *B* FROM WHOM *E. coli* 111;B4 WAS ISOLATED

Age groups of patients	No. of cases
Newborn	2
2 days to 7 days	12
7 days to 18 days	5
6 months to 9 months	5
One year	1
Total	25

TABLE III

MONTHLY RECORD OF ISOLATIONS, NEW PATIENTS, AND REPEAT SPECIMENS FROM WHICH *E. coli* 111;B4 WAS ISOLATED

Month	No. of isolations		No. of new patients		Repeats		No. examined	
	*A**	*B**	*A*	*B*	*A*	*B*	*A*	*B*
November	7	0	5	0	2	0	24	0
December	24	0	17	0	8	0	148	3
January	4	5	3	2	1	3	45	10
February	1	2	0	0	1	2	30	4
March	0	0	0	0	0	0	34	3
April	0	0	0	0	0	0	50	1

* *Hospital.*

Table III shows the number of isolations, the number of new patients, and the number of repeat isolations by the month. Antibiotic therapy was started in the middle of December and the changes in nursing and cleaning technique recommended by the team from the Department of Health were put into effect. From that time there was a pronounced decline in the number of isolations from new patients and in the number of isolations from repeat specimens. As will be seen by the table, there were no new patients in February; in March and April all examinations were negative.

Isolation and Identification

Cultural

The cultural methods described have been in use in these laboratories since 1951 for the isolation and recognition of pathogenic *E. coli*. They parallel very closely those of Charter and Taylor in 1952 (3).

Both anal swabs and fecal specimens were cultured on 5% sheep's blood agar and on MacConkey's bile salt agar as soon as they were received. All specimens were then subjected to the routine examination for *Salmonella* and *Shigella* organisms.

Serological Tests

Colonies from the blood plate were used for the serological tests whenever possible, as those from the MacConkey plate often showed smooth to rough variation.

The surface of a glass slide was divided into six squares with a china-marking pencil and a drop of pooled OB serum was placed in each square. The OB serum pool contains six *E. coli* OB sera, namely 111;B4, 55;B5, 26;B6, 86;B7, 125, and 126. A sweep of the confluent growth from a blood plate was suspended in a drop of the serum and stirred thoroughly. A positive test gave instantaneous agglutination in large clumps. When a positive reaction was obtained, single colonies were tested in the same manner and a portion of a colony giving a positive test was transferred to a veal agar slope for biochemical and further serological tests.

If no agglutination was obtained with one sweep, a second and third sweep were made. If these sweep tests were negative, single discrete colonies from different areas of the plate were tested. All positive colonies were tested in normal saline and acriflavine because Charter and Taylor found that the types of *E. coli* we were seeking did not agglutinate in acriflavine or saline (3). Colonies that agglutinated in pooled OB serum were then tested in the six single OB sera.

The tube agglutination method was used for the recognition of the O antigen. O suspensions were prepared from broth cultures, incubated overnight, and boiled for one hour. Incubation was in a 50° C. water bath, and the tests were read the following morning.

Living cultures of the strains isolated in this outbreak clumped heavily and instantaneously in pooled OB serum and also in 111;B4 specific serum. Occasionally colonies gave a fine agglutination in pooled OB serum. This type of colony usually clumped in acriflavin and could not be confirmed as positive by tube agglutination, either with living or boiled antigen.

Difficulty was experienced in obtaining a satisfactory H antigen. The cultures were sluggishly motile, when first isolated, but there did not appear to be a sufficient development of the H antigen to give visible agglutination in H antiserum. However, after serial transfer through semisolid agar the majority of the strains were agglutinated by *E. coli* H2 antiserum.

Biochemical Reactions

The variation in biochemical behavior of the six types of *E. coli* isolated in this laboratory since 1952 is shown in Table IV.

TABLE IV

VARIATION IN THE BIOCHEMICAL REACTIONS

Type	Glucose Maltose Mannitol Lactose Arabinose Xylose	Salicin	Rhamnose	Dulcitol	Sucrose	Inositol	H_2S	Indole	Gas	Urea	Hemolysis
111;B4;2	+	−	+	$+_3$	$+_3$	−	−	+	+	−	−
111;B4;12	+	$+_2$	$+_7$	$+_7$	− or + *L*	−	−	+	+	−	−
55;B5;2	+	−	+	$+_3$	−	−	−	+	+	−	+
55;B5;−	+	$+_3$	+	+	−	−	−	+	+	−	−
26;B6;⊕	+	$+_7$	−	−	$+_2$	−	−	+	+	−	−
26;B6;⊕	+	+	+	+	−	−	−	+	+	−	−
26;B6;⊕	+	$+_7$	+	−	$+_7$	−	−	+	−	−	−
86;B7;4	+	+	+	+	$+_{14}$	−	−	+	+	−	−
125;⊕;—	+	+	+	−	+	−	−	+	+	−	−
126;⊕;2	+	+	$+_5$*	−	−	−	−	+	+	−	−

⊕ *Not yet classified.* * *No gas formed.*
The numerals indicate the number of days before reaction becomes positive. L = Late.

All strains were negative in inositol. Two strains of 26;B6 were anaerogenic, and two of 55;B5 showed hemolysis on agar plates containing 5% sheep erythrocytes. There was variation in the fermentation of salicin both by 111;B4 and 55;B5 cultures.

Serological Tests

Table V shows the results of the serological tests on all strains isolated. All the 111;B4 cultures could be separated into the three groups described by Kauffmann (4). Thirty-three of the motile cultures contained H2 antigen, six had H12 antigen, and by far the largest proportion were non-motile and without H antigen.

In O group 55, three cultures were motile. The H antigen of these strains failed to agglutinate in H6 antiserum. Two of them were agglutinated to the titer of the H2 antiserum, and one to the titer of H4 serum. An antigen of the latter strain boiled for one hour did not agglutinate in any O antiserum, but when boiled for two and one-half hours it agglutinated in the 55 O serum to a dilution of 1 : 1600. The titer of this serum was 6400.

The No. 7 culture in the table agglutinated to the titer of the B5 antiserum. No O agglutination was obtained when the antigen was boiled for one hour, two and one-half hours, or when heated to 120° C.

All the strains of 26;B6 were actively motile, but the H antigen was not agglutinated by any of our available H antisera.

The H antigen of the one 86;B7 culture agglutinated to the titer of the H4 antiserum.

A further serological study of the O55, O26, and O86 strains is being carried out.

TABLE V

SEROLOGICAL BEHAVIOR OF THE *E. coli* STRAINS ISOLATED

Group	No. of strains	O antigen	K antigen	Motility	H antigen
1	33	111	B4	+	2
2	6	111	B4	+	12
3	117	111	B4	−	−
4	2	55	B5	+	2
5	1	55	B5	−	−
6	1	55	B5	+	4
7	1	—	B5	−	−
8	9	26	B6	+	⊕
9	1	86	B7	+	4
10	1	125	⊕	−	−
11	3	126	⊕	+	2

⊕ *Unclassified.*

TABLE VI

ANTIBIOTIC SENSITIVITY, SALICIN FERMENTATION, AND H ANTIGENS

Type	H antigen	Salicin fermentation	Antibiotic sensitivity tests							
			Streptomycin	Aureomycin	Chloromycetin	Terramycin	Polymixin B	Sulphadiazine	Sulphathiazol	Sulphamerazine
111;B4	2	−	*R*	*R* or *MS*	*S*	*S* or *MS*	*S*→*R*	*R*	*R*	*R*
111;B4	—	−	*R*	*R* or *MS*	*S*	*S* or *MS*	*S*→*R*	*R*	*R*	*R*
111;B4	12	+	*S*	*S* or *MS*	*S*	*S*	*S*	*S*	*S*	*S*
111;B4	12	+	*R*	*S*	*S*	*S*	*S*	*S*	*S*	*S*

S=*sensitive;* *MS*=*moderately sensitive;* *R*=*resistant.*

Antibiotic Sensitivity

Antibiotic sensitivity tests on type 111;B4;12 were conspicuously dissimilar to those on types 111;B4;2 and 111;B4;–. Those strains having the H2 antigen and those that were non-motile and without H antigen were uniformly resistant to streptomycin and the sulpha group, whereas those strains having the H12 antigen were sensitive to streptomycin and the sulpha group. The ability to ferment salicin was also a characteristic of the 111;B4;12 strains.

Table VI shows the sensitivity tests and salicin fermentation on 111;B4 types.

As is shown in this table one exception to the above observation was noted. One culture of *E. coli*, 111;B4;12, was resistant to streptomycin although it was salicin positive. Upon enquiry it was found that this child had been admitted to hospital for burns, and had received a nine-day course of penicillin and streptomycin in the two-week period prior to the onset of diarrhea.

These results confirm the observations of Modica, Ferguson, and Ducey (5) that salicin negative strains of 111;B4 are resistant to streptomycin whereas salicin positive strains are sensitive. In addition we observed that the salicin positive and streptomycin sensitive cultures had H12 antigen whereas those cultures that were salicin negative and streptomycin resistant had H2 or were non-motile and without H antigen. A large proportion of the non-motile cultures were isolated from the same outbreak as were the 111;B4;2 strains.

Acknowledgment

We wish to acknowledge the work of Mr. Carl Merger who carried out all the antibiotic sensitivity tests.

References

1. Bray, J. Isolation of antigenically homogeneous strains of *Bact. coli neapolitanum* from summer diarrhoea of infants. J. Pathol. Bacteriol. 57 : 239-247. 1945.
2. Bray, J. and Beavan, T. E. D. Slide agglutination of *Bacterium coli* var. *neapolitanum* in summer diarrhoea. J. Pathol. Bacteriol. 60 : 395-401. 1948.
3. Charter, R. E. and Taylor, J. Cultural and serological reactions of strains of *Bact. coli* isolated from babies. J. Pathol. Bacteriol. 64 : 729-734. 1952.
4. Kauffmann, F. and Dupont, A. *Escherichia* strains from infantile epidemic gastro-enteritis. Acta Pathol. Microbiol. Scand. 27 : 552-564. 1950.
5. Modica, R. I., Ferguson, W. W., and Ducey, E. F. Epidemic infantile diarrhoea associated with *Escherichia coli* 111;B_4. J. Lab. Clin. Med. 39 (1) : 122-128. 1952.
6. Neter, E. and Shunway, C. N. *E. coli* serotype D433. Occurrence in intestinal and respiratory tract, cultural characteristics, pathogenicity, sensitivity to antibiotics. Proc. Soc. Exptl. Biol. Med. 75 : 504-507. 1950.
7. Neter, E., Korns, R. F., and Trussell, R. E. Association of *Escherichia coli* serogroup O111 with two hospital outbreaks of epidemic diarrhoea of the newborn infant in New York State during 1947. Pediatrics, 12(4) : 377-383. 1953.
8. Ørskov, F. On occurrence of *E. coli* belonging to O group 26 in cases of infantile diarrhoea and white scours. Acta Pathol. Microbiol. Scand. 29 : 373. 1951.
9. Taylor, J. and Charter, R. E. The isolation of serological types of *Bact. coli* in two residential nurseries and their relation to infantile gastro-enteritis. J. Pathol. Bacteriol. 64(4) : 715-728. 1952.
10. Taylor, J., Powell, B. W., and Wright, J. Infantile diarrhea and vomiting, a clinical and bacteriological investigation. Brit. Med. J. 2 : 117-119. 1949.

A PRELIMINARY NOTE ON THE EXPERIMENTAL INFECTION OF CHICK EMBRYOS WITH *ENTAMOEBA INVADENS*[1]

By Eugene Meerovitch[2]

Abstract

Developing chicken embryos were infected intravenously with bacteria-free *Entamoeba invadens*. The parasites invaded the livers of the embryos, and caused the production of typical amoebic abscesses.

Introduction

The only attempt to infect chick embryos with parasitic amoebae known to the author is that of Everitt, Sadun, and Carrera (2), in which *Entamoeba histolytica* with associated bacteria were used to induce infections by various inoculation routes. Shaffer, Sienkiewicz, and Washington (7) have shown that *E. histolytica* will enter blocks of chick embryo tissue suspended in tissue-culture nutrient fluid. The author considered that *E. invadens*, which is being maintained in the Institute of Parasitology in bacteria-free cultures according to the method of Miller (4), should prove to be a favorable species with which to investigate amoebiasis in chick embryos. *E. invadens*, which morphologically resembles *E. histolytica*, is a parasite of reptiles in which it is as pathogenic as *E. histolytica* is in man (6). A host list for *E. invadens* showing both the spontaneous and experimental infections in snakes and lizards so far recorded is given by Osman Hill and Neal (5).

Materials and Methods

Two strains of *E. invadens*—the P.Z. and the I.P. strains—were used in these experiments. The amoebae from a culture selected for inoculation were concentrated by centrifugation and were used to infect about six embryos. No attempt was made in these investigations to count the number of organisms injected into each embryo.

Eleven to 15-day-old white Leghorn embryos were used. After inoculation with amoebae the embryos were kept at 30° C. This temperature permitted growth of the amoebae and survival of the embryos.

The intravenous method of inoculation described by Beveridge and Burnet (1) was used in the experiments reported in this paper. This method proved to be more satisfactory than the chorioallantoic, amniotic, or the yolk-sac method, because it afforded a means of studying the lesions produced by the amoebae within the embryo, rather than the invasiveness of the amoebae into the embryonic membranes or into the embryo.

[1] *Manuscript received October 15, 1954.*

Contribution from the Institute of Parasitology, McGill University, Macdonald College, P.Q., Canada, with financial assistance from the National Research Council of Canada and The Banting Research Foundation.

[2] *Lecturer and Research Assistant.*

The embryos were removed from the shell one to five days after inoculation and examined for gross pathology. Fragments of liver tissue were examined for the presence of amoebae and pieces of liver were fixed in Zenker's-formalin. Sections were stained in Heidenhain's iron haematoxylin, Ehrlich's haematoxylin – eosin, and in Weigert's iron haematoxylin – aniline-eosin – naphthol green B (3).

Results

Thirty-seven chick embryos were inoculated, 24 with the I.P. and 13 with the P.Z. strain of *E. invadens*. Of those infected with the I.P. strain, 11 (or 45.8%) were found to be harboring amoebae in the liver; of those infected with the P.Z. strain, eight (or 61.5%) were positive. The total number of positive embryos was 19 (or 51.3%).

Livers were examined either directly, as soon as the embryos were removed from the shell; by culturing in a suitable medium; or by histological examination after being stained with one of the stains mentioned above.

In most cases, livers harboring amoebae were hemorrhagic and patchy in gross appearance, while those that looked normal were negative. Of the 19 positive embryos, six had definite abscesses which were located on the left lobe of the liver in each case. Stained sections of these abscesses showed numerous vegetative amoebae among the liver cells. No cysts were found in the liver lesions. The amoebae contained host cell debris, some of which was partially digested (Fig. 1). Table I gives, in a concise form, the data pertinent to the 19 positive embryos.

Only the livers were examined in this preliminary study. It was assumed that, as the intravenous route of inoculation was used, the liver was the most likely organ to be infected.

More extensive experiments are being conducted and the results will be published in due course.

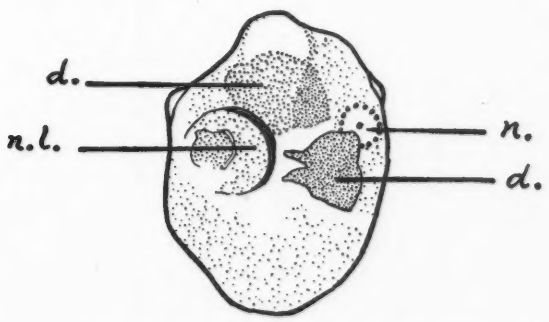

FIG. 1. A camera lucida drawing of *E. invadens* in a section of liver, showing ingested host cell debris. × 4500. *d*—host cell debris; *n.l.*—nucleus of liver cell, partially digested; *n*—nucleus of the amoeba.

TABLE I

Positive embryo numbers	38	40	43	45	46	48	54	55	56	58	59	64	65	66	70	71	72	79	82
I.P. strain	*	*	*	*	*	—	*	*	—	—	—	—	—	—	—	*	*	*	*
P.Z. strain	—	—	—	—	—	*	—	—	*	*	*	*	*	*	*	—	—	—	—
Age of embryo when inoculated (days)	15	15	12	12	12	12	11	11	12	12	12	14	14	14	14	14	14	13	13
Examined after number of days	3	1	2	2	2	2	2	3	3	3	4	3	3	3	4	5	5	4	1
Dead or alive at examination	*a*	*a*	*a*	*d*	*a*	*a*	*a*	*d*	*a*	*d*	*a*	*a*	*a*	*a*	*a*	*d*	*d*	*a*	*a*
Liver abscess present	—	—	—	—	—	—	—	—	—	—	—	—	+	+	—	+	—	+	+

References

1. BEVERIDGE, W. I. B. and BURNET, F. M. The cultivation of viruses and rickettsiae in the chick embryo. Med. Research Council (Brit.), Special Rept. Ser. No. 256. 1946.
2. EVERITT, M. G., SADUN, E. H., and CARRERA, G. M. Cultivation of *Endamoeba histolytica* in the chick embryo. Exptl. Parasitol. 2 : 141-146. 1953.
3. GRIDLEY, M. F. A stain for *Entamoeba histolytica* in tissue sections. Am. J. Clin. Pathol. 24 : 243-244. 1954.
4. MILLER, M. J. Bacteria-free *Entamoeba invadens*. Nature, 172 : 1192. 1953.
5. OSMAN HILL, W. C. and NEAL, R. A. An epizootic due to *Entamoeba invadens* at the gardens of the Zoological Society of London. Proc. Zool. Soc. London, 123 (4) : 731-737. 1954.
6. RATCLIFFE, H. L. and GEIMAN, Q. M. Spontaneous and experimental amebic infection in reptiles. Arch. Pathol. 25 : 160-184. 1938.
7. SHAFFER, J. G., SIENKIEWICZ, H. S., and WASHINGTON, J. E. The propagation of *Endamoeba histolytica* in tissue-bearing culture without accompanying bacteria or other microorganisms. Am. J. Hyg. 57 : 366-379. 1953.

NOTE

A METHOD FOR SEPARATING MIXED CULTURES OF SALMONELLAE

By W. R. Bailey and Rhoda Laidley

The method employed represents a further application of the Gard technique (1, 2), which was originally used as a means of isolating phases of diphasic motile salmonellae by inoculating a semisolid agar medium in which antiserum for one phase was incorporated. The homologous phase is immobilized at the site of inoculation and the other phase develops uninhibited. In the separation of mixed cultures of motile salmonellae the principle entertained is the immobilization or suppression of one component culture 'in toto', either by the use of antisera corresponding to the O complexes of the components of the mixture if the mixture consists of serotypes from different somatic groups of the Kauffmann–White antigenic schema, or by the use of H antisera corresponding to the phase or phases of the components of a mixture when such belong to the same somatic group but possess unrelated H antigen complexes.

Mixed cultures are usually recognized in the initial phases of serological identification by their multiple agglutination reactions in polyvalent antisera or later in group specific or monospecific O and H sera. Such reactions are used as an index in the selection of sera for the preparation of the serum agar. When, for example, a mixture is recognized by the establishment of two somatic complexes, two serum agar plates are prepared to contain antisera for each complex respectively. The antisera used must be free of interfering antibodies.

Small Petri culture dishes (60 × 15 mm.), normally employed in this laboratory for motility tests and phase suppression procedures in the routine identification of *Salmonella* cultures, are used. Appropriate antisera of titer 1 : 10,000 to 1 : 20,000 are mixed with semisolid agar (meat infusion with 0.25 to 0.4% agar) at 47° C. to give a final dilution of 1 : 150 to 1 : 300. The medium is dispensed into the small plates in 6- to 8-ml. amounts and allowed to gel. These plates are inoculated in the center or near the periphery with the growth from an agar slope of the mixed culture by means of a needle or small loop. Only a small amount of inoculum is required and it is applied to both plates containing the selected antisera. Following an incubation period of 12–24 hr. at 37° C., the plates are examined for swarms. The uninhibited component of the mixture usually gives definite swarms after 12-hr. incubation and in many cases will have grown completely through the medium after 24 hr. Growth is selected from the edge of a swarm, plated on plain agar, and checked for purity with the necessary antisera by macroscopic slide agglutination tests.

Table I offers an example of a mixed culture where the components belong to two different somatic groups, while Table II exemplifies a mixture of two serotypes in the same somatic group but possessing unrelated H antigen complexes. In each example the serological reactions of the mixture are shown together with the reactions obtained when the mixture has been cultivated on the respective serum agar plates.

TABLE I

SEPARATION OF A MIXTURE WHOSE COMPONENTS POSSESS UNRELATED SOMATIC COMPLEXES

Mixture	Agar plate	Agglutination reactions with antisera						Result
		IV	V	VII	i	m, t	1, 2	
Multiple reaction with O antisera "VI, VII" and "IV, V, XII"	Control (no serum)	+++	+++	+++	+++	+++	+	Mixed
	With IV, V, XII serum	−	−	++++	−	++++	−	Pure *S. oranienburg*
	With VI, VII serum	++++	++++	−	+++	−	+	Pure *S. typhi-murium*

TABLE II

SEPARATION OF A MIXTURE WHOSE COMPONENTS SHARE THE SAME SOMATIC COMPLEX BUT POSSESS UNRELATED H ANTIGEN COMPLEXES

Mixture	Agar plate	Agglutination reactions with antisera						Result
		VI, VII	VII	k	1,5	5	z_{29}	
Multiple reaction with H antisera k, z_{29}, and "1,5"	Control (no serum)	++++	+++	+++	++	++	+++	Mixed
	With k–1, 5 serum	++++	+++	−	−	−	++++	Pure *S. tennessee*
	With z_{29} serum	++++	+++	++++	++	++	−	Pure *S. thompson*

Attempting separation of mixed cultures by colony selection from an agar plate is a laborious and time consuming procedure and in many instances not successful. Separation by serological suppression has proved to be time saving and of practical value in this laboratory.

The method to date has proved quite useful in separating mixtures of types that possess unrelated somatic complexes, which are the ones most easily recognized, as well as for mixtures the components of which share the same

O complex but exhibit unrelated flagellar antigen complexes. In the event of a mixture, however, consisting of serotypes with similar O antigens and a common phase, separation is difficult and more of academic than of practical interest.

Such procedures can only be employed in laboratories having access to a wide range of antisera.

1. GARD, S. Ein Colistamm mit Salmonella-H-Antigen, zugleich ein Beitrag zur Frage der Definition der Salmonellagruppe. Z. Hyg. Infektionskrankh. 120 : 59. 1937.
2. GARD, S. Das Schwarmphanomen in der Salmonellagruppe und seine praktische Ausnutzung. Z. Hyg. Infektionskrankh. 120 : 615-619. 1938.

RECEIVED OCTOBER 1, 1954.
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NOTE

AN OCCURRENCE OF *LEUCOCYTOZOON SIMONDI* IN MANITOBA

By A. Savage and J. M. Isa

This note is to record a disease of domestic ducklings in Manitoba, associated with a heavy infestation of *Leucocytozoon simondi.* Since Wickware (9) described this condition in Ontario it has been dealt with by Chernin and Sadun (1), Clarke (2), Fallis, Davies, and Vickers (3), Hartman (4), Huff (5), and O'Roke (7). Its possible importance to the population of migratory ducks has been indicated, though, so far as we are aware, the parasite has not been recognized in North America as far West as the 97th meridian.

On July 19th, 1954, we received a dead five weeks' old White Pekin duck for autopsy. It was not strictly fresh, hence gross lesions were masked. Even so, the examination of a blood smear revealed an enormous number of leucocytozoa. Two days later a sick bird was obtained from the owner, who stated that of the 51 ducklings he had procured from a Winnipeg hatchery, 18 had died recently and all in the same way. They stopped eating and developed diarrhea. In a majority of cases, they lay on their backs and struggled in that position until exhausted.

The specimen was in fair condition but refused feed. It was friendly, vocal, and bright, though weak, and very disinclined to move. Hydremia was marked and clotting time obviously increased. Precise measurements of these features were not made. After a number of blood films had been prepared the specimen was killed for examination. Principal gross findings were: a dark, soft spleen more than one inch in diameter, an apparently enlarged heart (?), and a finely mottled liver. Portions of these organs were preserved for further study.

Under the microscope blood films revealed large numbers of leucocytozoa both as mature gametocytes and in various developmental stages. They warranted the assumption that the bird had been subjected to more than one severe attack by Simuliidae.

A visit to the premises on October 5th indicated that only seven of the original ducklings remained alive. They appeared to be normal. We were informed that there were no other flocks of ducks in the vicinity.

The Rat River adjoins the farm concerned. It is not a fast stream and is muddy. The location of this outbreak is in De Salaberry Municipality 40 miles south of Winnipeg.

Much of Manitoba is included in more than one important "migration route" of aquatic wild fowl. It contains an abundance of black flies. Within it leucocytozoa have been recognized in the blood of turkeys (8), geese (6), and now ducks. Because of these considerations one wonders why disease of this kind has not been more in evidence.

We express our thanks to the Zoology Department, University of Manitoba, and to the Health of Animals Branch, Canada Department of Agriculture. Members of their staffs undertook the field trips in connection with this episode.

1. CHERNIN, E. and SADUN, E. H. *Leucocytozoon simondi* infections in domestic ducks in Northern Michigan with a note on *Haemoproteus*. Poultry Sci. 28 : 890-893. 1949.
2. CLARKE, C. H. D. Some records of blood parasites from Ontario birds. Can. Field Naturalist, 60 : 34. 1946.
3. FALLIS, A. M., DAVIES, D. M., and VICKERS, M. A. Life history of *Leucocytozoon simondi* Mathis and Leger in natural and experimental infections and blood changes produced in the avian host. Can. J. Zool. 29 : 305-328. 1951.
4. HARTMAN, E. The asexual cycle in *Leucocytozoon anatis*. J. Parasitol. 15 : 178-182. 1929.
5. HUFF, C. G. Schizogony and gametocyte development in *Leucocytozoon simondi* and comparisons with *Plasmodium* and *Haemoproteus*. J. Infectious Diseases, 71 : 18-32. 1942.
6. MCTAVISH, W. B. Unpublished report to Manitoba Game & Fisheries Branch, 1951-52.
7. O'ROKE, E. C. A malaria-like disease of ducks caused by *Leucocytozoon anatis* Wickware. Univ. Michigan, School of Forestry and Conservation Bull. 4 : 1-44. 1934.
8. SAVAGE, A. and ISA, J. M. An outbreak of Leucocytozoon disease in turkeys. Cornell Vet. 35 (3) : 270. 1945.
9. WICKWARE, A. B. Is *Leucocytozoon anatis* the cause of a new disease in ducks? Parasitology, 8 : 17-21. 1915.

RECEIVED DECEMBER 3, 1954.
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Style, arrangement, spelling, and abbreviations should conform to the usage of this journal. Names of all simple compounds, rather than their formulas, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed.

Manuscripts should be carefully checked before they are submitted; authors will be charged for changes made in the proof that are considered excessive.

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An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required, except in Notes.

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References should be listed **alphabetically by authors' names,** numbered, and typed after the text. The form of the citations should be that used in this journal; in references to papers in periodicals, titles should be given and inclusive page numbers are required. All citations should be checked with the original articles, and each one referred to in the text by the key number.

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Contents